COMMUNICATION

Similar Effects of *c*9,*t*11-CLA and *t*10,*c*12-CLA on Immune Cell Functions in Mice

D.S. Kelley[a,*], J.M. Warren[a], V.A. Simon[a], G. Bartolini[a], B.E. Mackey[b], and K.L. Erickson[c]

[a]Western Human Nutrition Research Center, ARS, USDA, and Department of Nutrition, University of California, Davis, California, [b]Western Regional Research Center, Albany, California, and [c]Department of Cell Biology and Human Anatomy, University of California, Davis, California

ABSTRACT: Published results regarding the effects of CLA on immune cell functions have ranged from stimulation to inhibition. In those studies, a mixture of CLA isomers were used, and food intake was not controlled. We have examined whether the discrepancies in the results of earlier studies may be due to the lack of controlled feeding and whether the two isomers of CLA may differ in their effects on immune cell functions. Three groups of C57BL/6 female mice were fed either a control, *c*9,*t*11-CLA-, or *t*10,*c*12-CLA (0.5 wt%)-supplemented diet, 5 g/d, for 56 d. At the end of the study, the number of immune cells in spleens, bone marrows, or in circulation; proliferation of splenocytes in response to T and B cell mitogens; and prostaglandin secretion *in vitro* did not differ among the three groups. Both CLA isomers significantly increased *in vitro* tumor necrosis factor α and interleukin (IL)-6 secretion and decreased IL-4 secretion by splenocytes compared to those in the control group. Thus, the two CLA isomers had similar effects on all response variables tested. The discrepancies among the results from previous studies did not seem to be caused by the differences in the isomer composition of CLA used.

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CLA is a collective term for isomers of linoleic acid that have conjugated double bonds. Depending on the position and geometry of the double bonds, several isomers of CLA have been reported (1). Feeding a mixture of these isomers altered several indices of immune cell functions in animal models (2–11) but not in humans (12,13). Even in the animal models, the effects of dietary CLA on immune cell functions have been modest and variable. Thus, in one study splenocyte proliferation in response to the T cell mitogen phytohemagglutinin (PHA) was enhanced 3 wk after supplementing mouse diets with CLA, but not after 6 wk (4). Proliferation in response to another T cell mitogen, Concanavalin A (Con A), was not altered at 3 and 6 wk after CLA supplementation. In another study, 8 wk of CLA supplmentation of young mice enhanced splenocyte proliferation at Con A concentrations of 0.5 and 5.0 mg/L, but not at 1.5 mg/L; CLA supplementation did not alter splenocyte proliferation in response to PHA (5). Similar inconsistencies were found regarding the effects of CLA feeding on cytokine secretion, delayed hypersensitivity skin response, and other indices of immune response (2–11). None of these studies controlled food intake or fed purified isomers of CLA, which may be partially responsible for some of the discrepancies. This study examined the effects of two purified isomers of CLA on immune cell functions under conditions of controlled food intake. Isomers used in our study were the *cis*-9 and *trans*-11 (*c*9,*t*11-CLA), and *trans*-10 and *cis*-12 (*t*10,*c*12-CLA), which are among the most abundant isomers found in the mixtures used in previous studies. We used a single concentration (0.5 wt%) of each isomer, which is equivalent to the concentrations of these isomers present in the CLA mixtures (0.1 to 1.5%) used in previous studies.

MATERIALS AND METHODS

Diets and animals. Highly enriched *c*9,*t*11-CLA, and *t*10,*c*12-CLA isomers as FFA were a kind gift from Natural ASA (Hovdebygda, Norway). Both preparations contained more than 85% of the isomer of interest. AIN-93G mouse diet was used as the basal diet. Fat source in the diet was corn oil, 50 g/kg. CLA isomers were added at 5 g/kg by replacing an equivalent amount of corn oil. Diets were constantly flushed with nitrogen gas while being gently mixed in a blender and then stored at −20°C. Fresh dietary packets were served each day.

Thirty-five 8-wk-old, pathogen-free C57BL/6N female mice were purchased from Charles River (Raleigh, NC) and divided into three groups (11 or 12/group). Animals were maintained in a sterile air curtain isolator at 25°C, with light and dark cycles of 12 h each. Based on our previous experience with food required by the mice in this age range (14), they were offered fresh food every day (5 g/animal/d) for 56 d. All food was consumed within 4–6 h in all groups. This avoided the need for paired feeding and reduced the possibility of CLA oxidation. At the end of the study, body weight of the mice fed the diet containing *t*10,*c*12-CLA was significantly lower than that of the other two groups, which did not differ from each other. All conditions and handling of the animals were approved by the Animal Care and Use Committee at the University of California–Davis.

*To whom correspondence should be addressed at USDA/ARS/WHNRC, Department of Nutrition, University of California–Davis, One Shields Ave., Davis, CA 95616. E-mail: dkelley@whnrc.usda.gov

Abbreviations: BrdU, 5-bromo-2-deoxyuridine; *c*, *cis*; Con A, Concanavalin A; DME, Dulbecco's modified Eagle medium; IL, interleukin; LPS, lipopolysaccharide; PGE_2, prostaglandin E_2; PHA, phytohemagglutinin; *t*, *trans*; TNFα, tumor necrosis factor α.

Tissue collection and cell culture. Mice were anesthetized, and blood was collected into EDTA-containing syringes by heart puncture. Spleens were aseptically removed, weighed, and placed in 5 mL Dulbecco's modified Eagle medium (DME; Sigma Chemical Co., St. Louis, MO) containing 5% FCS, 25 mmol Hepes, 8 mmol glutamine, penicillin 10 KU/L, and streptomycin 10 mg/L. Splenocytes were prepared as previously reported (15) and suspended in the DME medium containing 5% FCS. For cell proliferation assays, splenocyte concentrations were adjusted to 2 million/mL and 100 μL cell suspensions were added to each well of 96-well plates (Falcon Labware, Gaithersburg, MD). This was followed by the addition of an extra 100 μL media containing either the T cell mitogen, Con A (final concentrations 0, 0.5, 1.0, and 2.0 mg/L), or the B cell mitogen, lipopolysaccharide (LPS) (final concentrations 0, 2.0, 5.0, and 15.0 mg/L). Three wells were used for each mitogen concentration. Plates were then incubated at 37°C, 5% CO_2, and 100% humidity for 72 h. Proliferation was determined by incorporation of 5-bromo-2-deoxyuridine (BrdU) into the DNA (16,17); the Proliferation ELISA BrdU chemiluminescence kit was purchased from Roche (Mannheim, Germany) and used as described by the manufacturer. Results are expressed as relative light units (rlu)/s/well; one rlu is equivalent to one million photons/s/well.

For cytokine and eicosanoid secretion assays, 2 million splenocytes in duplicates were incubated in 1.0 mL of media containing LPS (0, 1, and 10 mg/L) or Con A (0, 1, and 5 mg/L) in 24-well plates for 4, 24, and 48 h. The cell culture media were collected following centrifugation and stored frozen at –70°C. Media from LPS-treated cultures were used to monitor tumor necrosis factor α (TNFα), interleukin (IL)-6, and prostaglandin (PG) E_2, while those from Con A-treated cells were used to determine IL-2 and IL-4 using ELISA assays. Mitogen concentrations and incubation times were selected based on preliminary experiments. ELISA kits for TNFα, IL-2, IL-4, and IL-6 were purchased from BD Pharmingen (San Diego, CA) and those for PGE_2 from Cayman (Ann Arbor, MI).

Statistical analysis. The data were subjected to one-way ANOVA between and within diets, using the SAS software (18). The one-tailed version of Dunnett's test was used to make comparisons of the test diet means with the control diet. Levene's test was used to examine the homogeneity of variance assumption. When heterogeneity was encountered, it was incorporated into the model using the SAS PROC MIXED.

RESULTS

Effect of CLA on cell numbers and splenocyte proliferation. Both CLA isomers did not alter the total number of circulating white blood cells or their percentages as neutrophils, lymphocytes, monocytes, eosinophils, and basophils; nor did they alter the number of splenocytes or bone marrow cells (not shown). Figure 1 shows that neither of the CLA isomers altered splenocyte proliferation in response to both the T and B cell mitogens, although the proliferation was increased with increase in mitogen concentration in all groups.

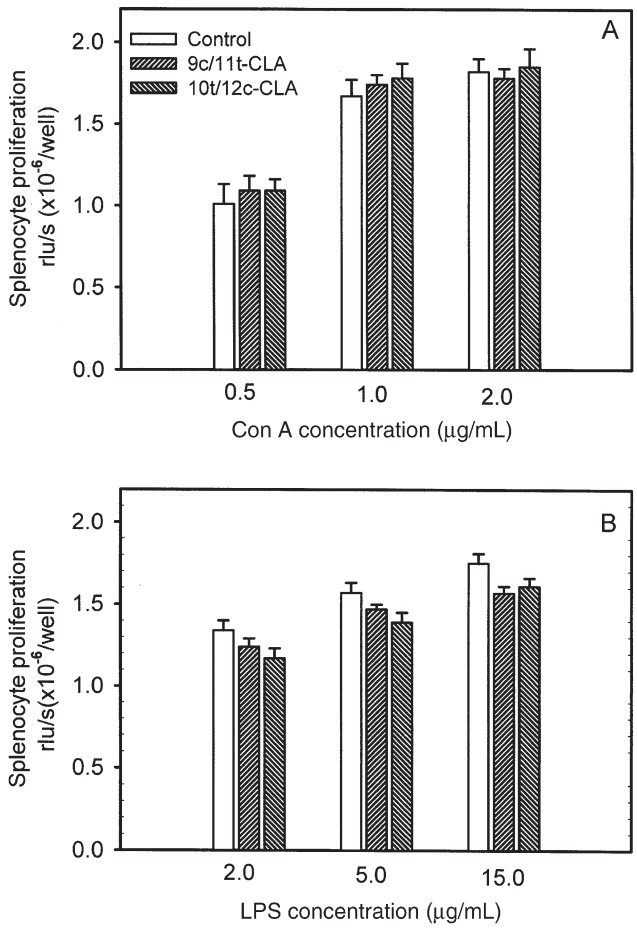

FIG. 1. Effect of CLA on splenocyte proliferation in response to Concanavalin A (Con A) (A) and lipopolysaccharide (LPS) (B). Splenocytes were cultured with the indicated mitogen concentrations for 72 h. Lymphocyte proliferation was examined by the incorporation of 5-bromo-2-deoxyuridine into the cellular DNA during the last 24 h in culture. Results are expressed as mean ± SEM of rlu (relative light units)/s/well. Neither of the CLA isomers altered splenocyte proliferation in response to both the mitogens.

Effect of CLA on eicosanoid and cytokine secretion. We examined the effects of CLA feeding on markers for inflammation (PGE_2, TNFα, and IL-6) and on representative cytokines for Th1 (IL-2) and Th2 (IL-4) cells. Treatment effects at the two mitogen concentrations were similar, but results are shown only for the higher mitogen concentrations (LPS, 10 μg/mL, for PGE_2, TNFα, and IL-6; Con A, 5 μg/mL for IL-2 and IL-4). Concentrations of PGE_2 in the medium collected at 4, 24, and 48 h from the splenocytes isolated from the mice fed control diet were 5.0 ± 0.6, 5.4 ± 0.5, 5.3 ± 0.6 pg/mL, respectively. The PGE_2 concentrations in the two CLA groups did not differ from those in the control group (not shown). Medium concentrations of TNFα and IL-4 decreased between 4 and 48 h, whereas those of IL-2 and IL-6 increased (Figs. 2A–D). TNFα secreted within 4 h of mitogen stimulation was significantly greater ($P < 0.001$) in both the CLA groups than in the control group. Medium concentration of TNFα did not differ among the three groups at 24 and 48 h, and also

between the two CLA isomers at all three time points. IL-6 secreted into the medium after 4 h of LPS treatment was nondetectable in all three groups. Twenty-four hours after LPS treatment, secreted IL-6 was significantly ($P < 0.01$) greater in both the CLA groups than in the control group, whereas after 48 h, the difference was significant only for *t*10,*c*12-CLA ($P < 0.01$) (Fig. 2B).

The concentration of secreted IL-2 increased six- to sevenfold from 4 to 24 h in all three groups of cells but did not change between 24 and 48 h; there was no difference in its concentration among the three groups at all three time points (Fig. 2C). Neither of the CLA isomers altered the concentration of IL-4 secreted within 4 h of mitogen treatment, but it was significantly reduced by both the isomers at 24 and 48 h after mitogen treatment (Fig. 2D). There was no difference in IL-4 concentrations in the two CLA groups at all three time points.

DISCUSSION

We compared the effects of feeding two purified isomers of CLA on several indices of immune and inflammatory responses in mice maintained on a controlled feeding regime. Effects of the two isomers on immune cell functions did not differ. Both the *c*9,*t*11-CLA and *t*10,*c*12-CLA did not alter the number of immune cells in circulation, in spleens, or in bone marrows. Neither of the isomers affected splenocyte proliferation in response to T and B cell mitogens, or the secretion of PGE_2 and IL-2. We had found similar results with feeding of a mixture of CLA isomers to healthy women (12,13). These results are in part consistent with those of earlier reports in mice (4,5). In one of these reports, 8 wk of CLA supplementation resulted in a modest increase in splenocyte proliferation at Con A concentrations of 0.5 and 5.0, but not

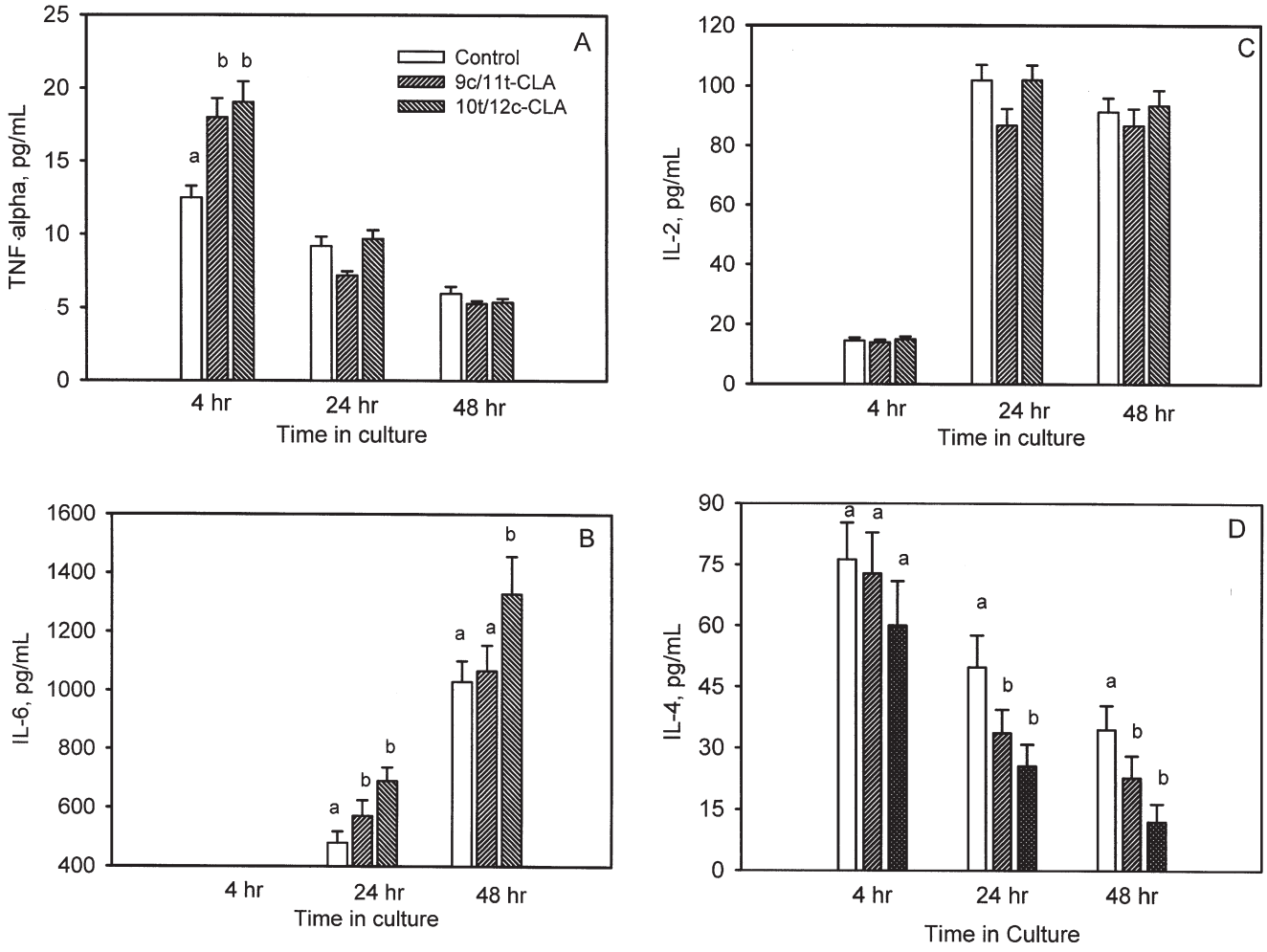

FIG. 2. Effect of dietary CLA on *in vitro* secretion of tumor necrosis factor α (TNFα) (A), interleukin (IL)-6 (B), IL-2 (C), and IL-4 (D) by splenocytes stimulated with 10 µg/ mL LPS (A and B) and 5 µg/mL Con A (C and D). Bars with different letters are significantly different. Secreted TNFα at 4 h was significantly ($P < 0.001$) greater in both CLA groups than in the control group; at 24 and 48 h there was no significant difference among all three groups. Secreted IL-6 at 24 h was significantly ($P < 0.01$) greater in both CLA groups than the control group; at 48 h the difference was significant only for the *t*10,*c*12-CLA group. At all times, secreted IL-2 was not significantly different among the three groups. Secreted IL-4 was significantly less in both CLA groups than in the control group at 24 and 48 h ($P < 0.03$) but not at 4 h.

at 1.5 mg/L (5). In the other report, 6 wk of CLA supplementation did not affect splenocyte proliferation (4). IL-2 secretion was increased in one (5) but not in the other study (4). Results regarding splenocyte proliferation differ from those of another study that found increased proliferation in response to both Con A and PHA in splenocytes isolated from mice fed a CLA-containing diet (2).

Both CLA isomers increased the secretion of TNFα and IL-6 and decreased that of IL-4 (Figs. 2A–D). These changes could have been easily missed if we had tested all cytokines only at a single time point. Our results regarding TNFα and IL-6 differ from those obtained with rat peritoneal macrophages, where feeding a mixture of CLA isomers decreased the secretion of both these cytokines (6). Those investigators reported that the effect of CLA varied with the n-6/n-3 FA ratio of the experimental diets. Results of our current study also differ from those found in humans (12,13), which most likely are due to the amount of CLA fed; the amount of CLA/kg body weight was 20 times greater in the mouse than in the human study. The amount of CLA needed in humans to match that used in the mice will require 75 g/d/60 kg person; this is greater than or equal to the total daily fat consumed by most humans and cannot be considered as a nutritional supplement.

Published effects of CLA on immune cell functions varied not only for the functions tested in this study but also for other functions including delayed type hypersensitivity response, ratios between helper and suppressor T cells, and serum immunoglobulin levels (4–10) as well. The results have ranged from stimulation to inhibition or no effect. These discrepancies do not seem to be due to the differences in isomer composition of the CLA mixtures used, because the effects of two isomers in the present study were similar. These may be due to lack of controlled feeding or other factors, including culture conditions (serum type and concentration, mitogen concentration and culture period), cell type (peritoneal vs. splenocyte vs. blood cells) composition, and storage of the diets, species, and age of the animals.

In conclusion, both CLA isomers have similar effects on several aspects on immune cell functions in mice; many indices were not altered by both isomers, whereas others were modestly affected by both isomers. Because of only modest effects, even at high concentrations of CLA intake, any clinical benefit to enhance immune status with nutritional supplements of CLA seems unlikely.

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